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EXPERIMENTAL ASSESSMENT OF A NEW, LOW-COST ANTIVENOM FOR TREATMENT OF CARPET VIPER (*ECHIS OCELLATUS*) ENVENOMING

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G. D. Laing, L. Lee, D. C. Smith, J. Landon and R. D. G. Theakston. Experimental assessment of a new, low-cost antivenom for treatment of carpet viper (*Echis ocellatus*) envenoming. *Toxicon* **33**, 307–313, 1995.—Morbidity and mortality due to envenoming by the carpet viper (*Echis ocellatus*) in northern Nigeria remains unacceptably high and constitutes a severe economic and public health problem to the local farming community in particular. The only effective treatment of systemic envenoming is antivenom, but supplies are very limited as the little that is available is either too expensive, ineffective or both. Here, we describe a new ovine antivenom, designed both to be effective and to be available at low cost. The antivenom, a polyclonal ovine Fab preparation, provides superior protection, both *in vivo* and *in vitro*, to the best alternatives, the monospecific South African Institute of Medical Research antivenom and the polyspecific Pasteur Isper Africa antivenom. Fab fragments, which have the advantages of large volumes of distribution and, theoretically, low immuno-reactivity, are produced by a reusable solid-phase papain matrix which eliminates enzyme contamination of the product and reduces cost. The antivenom is lyophilised for increased stability and extended shelf-life in tropical climates where it is often impossible to keep such products cool.

INTRODUCTION

Snakebite continues to be a major medical problem among rural communities of the savanna region of West Africa, including Nigeria. The saw-scaled or carpet viper (*Echis ocellatus*) is the most important cause of snakebite mortality and morbidity in this region, with farmers and children being particularly at risk. The main clinical features of *E. ocellatus* envenoming are haemorrhage, incoagulable blood, hypovolaemic shock and local swelling, bleeding and necrosis. Untreated mortality in Nigeria reaches 10–20% (Warrell and Arnett, 1976; Pugh and Theakston, 1980, 1987; Pugh *et al.*, 1979) and, in some rural hospitals, 50% of the total bed capacity may be occupied by snakebite victims at peak times.

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The lack of availability, cost, lability and poor neutralising potency of antivenom in recent years has prohibited effective treatment in the majority of patients. Here we report on the production and preclinical assessment of a novel ovine Fab-based antivenom designed to be safe and effective and, equally importantly, to have low manufacturing costs and prolonged stability at ambient tropical temperatures. Recently, the development of Fab antivenoms raised against the venoms of other snake species has been reported by our group (Smith *et al.*, 1992; Rawat *et al.*, 1994).

Groups of sheep were immunised monthly with small amounts of *E. ocellatus* venom, and Fab fragments were then prepared from pools of immune serum utilising a reusable solid-phase papain matrix. The efficacy of the ovine Fab antivenom was compared with that of the best (though unavailable) alternative, the monospecific equine 'Echis' antivenom produced by the South African Institute for Medical Research (SAIMR). The Behringwerke North and West Africa polyspecific antivenom and the Pasteur Ipser Africa polyspecific equine antivenom, currently in restricted use in Nigeria (Theakston and Warrell, 1991), were also studied. The Fab antivenom was assessed both *in vivo* and *in vitro* using standard WHO tests (Theakston and Reid, 1983; WHO, 1981).

MATERIALS AND METHODS

Venom

Venom was obtained by pooling the monthly milkings from 200 adult specimens of *E. ocellatus* held in captivity at the Liverpool School of Tropical Medicine. These snakes were captured from the wild in Kaltungo, Bauchi State, Northern Nigeria.

Preparation of Fab Echis antivenom

Immunisation and bleeding. Flocks of Welsh half-bred ewes were immunised using a maximum of 1 mg of whole venom per month (Sidki *et al.*, 1987). Thereafter, blood (10 ml per kg body weight) was collected at monthly intervals (Smith *et al.*, 1992) and the serum aspirated using a sterilising filter (0.2 μm) and stored frozen at -20°C until required.

Determination of antivenom titre. Antivenom samples and bleeds were assessed as described previously (Smith *et al.*, 1992) using enzyme-linked immunosorbant assay (ELISA) dilution curves and small-scale affinity purification (Fig. 1).

Commercial antivenom samples (SAIMR, Pasteur and Behringwerke) were also assessed by small-scale affinity purification to determine specific antibody concentrations (Table 1). Phenol, included as a preservative in commercial preparations, was removed by dialysis against phosphate-buffered saline (PBS) before application to affinity columns. This treatment did not result in loss of protein as judged by optical density and ELISA assessment.

Immunoglobulin purification and Fab production. Ovine serum pools were made from immune sheep blood 30 weeks after primary immunisation. The serum immunoglobulin fraction was then purified by salt precipitation, washed twice and, after centrifugation to pellet the precipitate, resuspended in 0.9% saline (Rawat *et al.*, 1994). The solution was then diafiltered against a 10 kDa Membrane (Sartorius 'UltraSart Module', Polysulphone), concentrated to approximately 50 g/litre and finally sterile filtered (SartoBran pH, 0.2 μm).

Digestion of the purified immunoglobulin was performed using a solid-phase papain matrix in the presence of L-cysteine (1.6%). The digestion matrix comprised sterile, depyrogenated papain covalently linked, via an amine linkage, to activated cellulose. Details of the manufacture and validation of this matrix will be given elsewhere. The cellulose–papain matrix was incubated with immunoglobulin until complete digestion of the IgG (as assessed by gel filtration FPLC assessment; Rawat *et al.*, 1994) was achieved. Digestion was terminated by centrifugation of the mixture to pellet the matrix which could then be reused up to eight times for subsequent batches. Iodoacetamide solution (2.8% in water) was then added 1:10 to the Fab solution. The Fab, as the supernatant, was diafiltered and washed (12 volumes) against saline to concentrate the antivenom and bring cysteine, iodoacetamide and sodium sulphate levels to below 1 ppm. It was then sterile filtered and lyophilised.

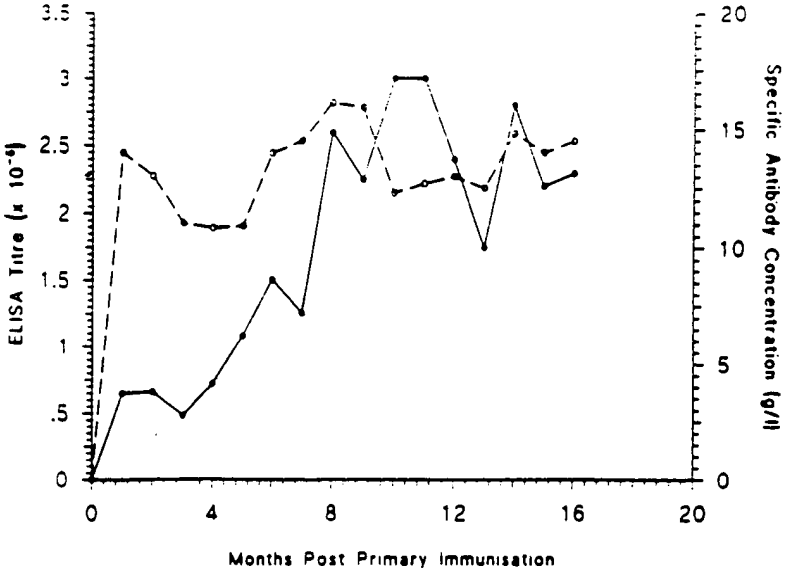

Fig. 1. Immune response of sheep immunised with *Echis ocellatus* venom. ELISA titres (—●—) and specific antibody concentration (—○—) of serum samples from sheep immunised with 1.0 mg of whole venom per month. Sheep were bled 2 weeks after each immunisation. Each point represents the mean of three determinations; the coefficient of variation was 7%.

Antivenoms

The following antivenoms were compared experimentally with the lyophilised ovine Fab product (Therapeutic Antibodies Inc., monospecific Echis antivenom, raised against the venom of Nigerian *E. ocellatus*, reconstituted to 10 ml with sterile water; Batch no. 004/50/001, lot no. 148., expiry 1/7/95).

(1) SAIMR. Monospecific Echis antivenom raised against the venom of Kenyan *E. pyramidum* (equine F(ab)$_2$, liquid 10 ml; Theakston and Warrell, 1991). Batch no. A5724, expiry 8/93 clear.

(2) Pasteur Ipser Africa polyspecific antivenom raised against the venoms of the puff adder (*Bitis arietans*), gaboon viper (*B. gabonica*), *E. carinatus*, Egyptian cobra (*Naja haje*), forest cobra (*N. melanoleuca*), spitting cobra (*N. nigricollis*) and mamba (*Dendroaspis* spp.) (equine F(ab)$_2$, liquid 10 ml; Theakston and Warrell, 1991), lot no. J5147, manufactured 22 2 93, expiry 3/96 clear.

(3) Behringwerke North and West Africa polyspecific antivenom raised against the venoms of *B. gabonica*, *E. carinatus* (East Africa), *N. haje*, *Vipera lebetina* (Levantine viper), *B. arietans*, *N. nigricollis* and possibly other species (equine F(ab)$_2$, liquid 10 ml; Theakston and Warrell, 1991), lot no. 207045, expiry 16/11 91, clear.

Antivenom purity assessment. The sterility, endotoxin content and homogeneity of the ovine Fab and the equine (SAIMR, Pasteur and Behringwerke) antivenoms were determined using the USP sterility test (United State Pharmacopoeia, 1980), the LAL gel clot endotoxin test (United States Pharmacopoeia, 1980) and gel filtration FPLC, respectively (Rawat *et al.*, 1994) (Table 1, Fig. 1).

Table 1. Antivenom characterization

Antivenom	Sterility	Endotoxin concentration (Eu ml)	Protein concentration (g/litre)	Specific antibody* (%)
Ovine Fab	Sterile	<0.25	50.1	30.0
SAIMR	Sterile	0.5–1.0	120.3	18.6
Pasteur	Sterile	2.0–4.0	52.8	3.6
Behringwerke	Sterile	0.5–1.0	64.5	10.9

* Specific antibody, active against venom components, was determined using small-scale affinity purification as described by Smith *et al.* (1992). See Materials and Methods section.

Eu, Endotoxin units.

Protein concentration determination of antivenoms. The protein concentrations of all four antivenoms were determined using optical density measurements at 280 nm (Table 1).

In vitro antivenom potency assessment. The minimum coagulant dose of the venom pool, defined as the least amount of venom in mg dry weight which clots a standard citrated solution of human plasma (MCD-P) in 60 sec at 37 C, was determined (Theakston and Reid, 1983). The ability of each of the four antivenoms to neutralise the procoagulant effect of one MCD-P was then assessed (Laing *et al.*, 1992). The effect of fibrinogen (MCD-F) was not determined as *E. ocellatus* venom does not contain a thrombin-like enzyme (Theakston and Reid, 1983).

In vivo antivenom potency assessments. The minimum haemorrhagic dose (MHD) in rats, the minimum necrotising dose (MND) in rats, the minimum defibrinogenerating dose (MDD) in mice and the i.v. LD_{50}, also in mice, were determined for the venom pool as described by Theakston and Reid (1983).

The neutralising ability of each of the three antivenoms against one MHD, one MND, one MDD and 5 × i.v. LD_{50} was evaluated as described by Laing *et al.* (1992).

For both *in vitro* and *in vivo* neutralisation assays, venom and antivenom were mixed and incubated for 30 min at 37 C prior to addition to the assay system (Laing *et al.*, 1992).

RESULTS

ELISA titres and venom-specific antibody concentrations

All sheep immunised with *E. ocellatus* venom responded well (Fig. 1). Small-scale affinity purification of the individual immune sera, carried out using the method of Smith *et al.*, (1992), suggested very high levels of specific antibody (11–15 g litre) which were obtained after only 6 weeks of immunisation and remained roughly constant throughout the study.

In contrast, ELISA titres appeared to rise slightly during the study period suggesting a progressive increase in antivenom avidity (Steward and Lew, 1985).

Assays of venom potency and antivenom efficacy

The intravenous LD_{50} of *E. ocellatus* venom was 20.1 μg/mouse (95% confidence limits, 17.3–22.8 μg/mouse). The MHD was 9.0 μg/rat, the MND 30 μg/rat, the MDD 4.0 μg/mouse and the MCD-P 4.0 μg/ml. All results, apart from the LD_{50}, were the mean of two assays. The venom was strongly haemorrhagic, had a powerful procoagulant effect causing fibrinogen consumption and possessed a fairly high level of necrotising activity. It was generally similar to earlier batches of Nigerian *E. ocellatus* venom tested (Theakston and Reid, 1983).

Table 2 shows the neutralising efficacy of the four different antivenoms against both lethal and other biological venom effects (haemorrhage, necrosis, defibrinogenation and procoagulant effect on plasma), given in microlitres and in micrograms of antivenom. The levels given in mass units are more realistic for the purpose of direct comparison of the antivenoms, but for clinical comparison it is the volume which is actually more meaningful. The most important parameter was the life-saving efficacy against the lethal effect of 5 × LD_{50} of the whole venom. The Fab antivenom possessed about the same overall potency as the SAIMR monospecific antivenom in terms of micrograms of Nigerian *E. ocellatus* venom neutralised per microlitre of antivenom but, weight for weight, the Fab antivenom was more than twice as effective as the SAIMR antivenom (Table 2). The Pasteur and Behringwerke polyspecific antivenoms were not significantly different to each other in their efficacy in neutralising venom lethality (as judged by overlap of the 95% confidence limits), but they were approximately 5-fold and 2-fold less active than the Fab and SAIMR antivenoms, respectively (Table 2).

Table 2. Neutralising efficacy of four antivenoms active against Nigerian *Echis ocellatus* venom (results in μl and μg antivenom)

	ED_{50}* (mouse)	Neutralisation of: MHD (rat)	MND (rat)	MDD (mouse)	MCD-P (ml)
Fab antivenom					
μl	12.99 (10.64–14.83)	2.0	10.0	5.0	3.0
μg	651 (533–743)	100	501	251	150
Pasteur antivenom					
μl	54.56 (40.60–68.57)	10.0	50.0	50.0	15.0
μg	2881 (2144–3620)	528	2640	2640	792
SAIMR antivenom					
μl	12.77 (10.30–16.20)	1.0	5.0	5.0	7.0
μg	1536 (1239–1949)	120	602	602	842
Behringwerke antivenom					
μl	70.31 (53.34–98.69)	12.4	25.4	10.6	20.2
μg	4535 (3440–6366)	780	1638	684	1303

95% confidence limits in parentheses.
Results are the mean of two assays.
* The ED_{50} was calculated against 5 × i.v. LD_{50} venom (see Materials and Methods section).

These findings were reflected in the neutralisation of other venom activities by the ovine Fab antivenom, which was slightly more effective than the SAIMR antivenom in neutralising individual venom activities (e.g. haemorrhage, necrosis, defibrinogenation and plasma coagulant) and about five times more active than the Pasteur and 8 times more active than the Behringwerke antivenom in neutralising venom haemorrhagic activity (Table 2). Other results on neutralisation of venom necrotising, procoagulant and *in vivo* defibrinogenating activities gave similar results. Overall, the order of efficacy for neutralisation of lethal and other activities was Fab > SAIMR > Pasteur > Behringwerke (Table 2).

DISCUSSION

There are three criteria that a successful antivenom must meet. First, it must be safe and not elicit the relatively high incidence of both early and late reactions associated with most equine venom antibodies (Lockey *et al.*, 1987; Sutherland, 1977; Sutherland and Lovering, 1979). Thus the product should contain only very low levels of pyrogen, must be sterile and should not activate complement. Although with prudent processing, sterile, low endotoxin content products can be made, the best equine F(ab)$_2$ antivenoms are still associated with a 6–7% incidence of side-effects, due primarily to an inherent sensitivity to equine protein in many populations (Lamson, 1924) and the ability of divalent F(ab)$_2$ to cross-link antigen (Karlson-Stiber and Persson, 1994). The Fab-based antivenom described here is designed to overcome these problems by using sheep as the source animal and Fab as the active ingredient, the univalency of which prohibits mixed complex formation (Cartilage *et al.*, 1992).

Second, an antivenom must be affordable by its intended market. Thus, in developing countries where the need is greatest, high-cost products will not be available to the general population. The Fab antivenom is designed to be produced at minimum cost and the use of sheep as a host animal has been described elsewhere (Sjostrom *et al.*, 1994); these animals are cheaper to keep than the horse, produce high-titre, high-affinity antibodies quickly and, as many can be immunised and only small amounts of venom are needed.

are more cost effective. Side-effects are best reduced by efficient processing and enzyme digestion and removal of the Fc fraction of the IgG molecule (Smith *et al.*, 1994). This can be costly and we have employed a single phase method whereby the enzyme may be reused, which reduces costs.

Finally, the product must be efficacious. Results of the potency assays indicate the superiority of the Fab monospecific Echis antivenom in all respects. The most meaningful assay is the ED_{50} determination (Table 2), which is a measure of the overall efficacy of the antivenom against whole venom, and is a direct measure of its life-saving potential. In this, as in the other assays, the monospecific antivenoms (Fab and SAIMR) proved vastly superior to the polyspecific products (Pasteur and Behringwerke): this is not surprising since the monospecific antivenoms are composed of antibodies directed against a single venom species (Christensen, 1966). The Fab monospecific antivenom, both on a volume/weight and on a weight/weight basis, more effectively neutralised the overall lethal effect of *E. ocellatus* venom, and was generally more active than the SAIMR monospecific antivenom when neutralising other venom activities (Table 2). This superiority is probably explained by the fact that the Fab antivenom was prepared against the same pool of Nigerian *E. ocellatus* venom as that against which it was tested, whereas SAIMR Echis antivenom is prepared using a pool of Kenyan *Echis* venom, possibly *E. pyramidum* (Theakston and Warrell, 1991), from a different geographical area of Africa.

Although it is tempting to predict clinical efficacy from these results, the findings may or may not correlate with the clinical observations and may not be infallible predictors of clinical efficacy (Warrell *et al.*, 1980; Keegan *et al.*, 1964, 1965). Nevertheless, many studies have demonstrated a high degree of correlation. For example, Warrell *et al.* (1986), testing three monospecific antivenoms against Malayan pit viper (*Calloselasma rhodostoma*) venom in southern Thailand, reported good agreement between clinical observation and experimental results, and Laing *et al.* (1992), using three Brazilian antivenoms, demonstrated that preclinical findings reflected the excellent clinical results (Cardoso *et al.*, 1993).

In conclusion, the assays carried out on the four antivenoms demonstrated that experimentally at least, the response to the Fab antivenom was the best, followed by the second monospecific Echis antivenom tested. The polyspecific preparations were significantly less potent. The only truly realistic assessment of such antivenoms, however, is to perform a properly controlled clinical comparative trial using, ideally, the same batches of antivenom. A comparative trial of the same batches of the Fab and the Pasteur Ipser Africa antivenoms is currently being carried out in Kaltungo, Nigeria, and will be the subject of a future publication.

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